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PREFACE

The present thesis was made in collaboration between Environment & Resources DTU (E&R DTU) and DHI – Water and Environment. Copenhagen Energy participated as third party. The work was carried out between September 1997 and May 2001 as an Industrial Ph.D. project under the Danish Academy of Technical Sciences funded by DHI - Water and Environment and The Danish Agency for Development of Trade and Industry.

The thesis consists of a) a summary of the project related literature, b) three papers submitted to international peer-reviewed periodicals, and c) one manuscript in preparation. The papers are not included in the this electronic version but can be obtained from the Library at Environment & Resources DTU, Bygningstorvet, Building 115, Technical University of Denmark, DK-2800 Kgs. Lyngby (library@er.dtu.dk).

A) Boe-Hansen, R., Albrechtsen, H. -J., Arvin, E. and Jørgensen, C. (2002a) Development of a model distribution system for studies of microbial activity and changes in drinking water quality. *Manuscript in preparation*.

B) Boe-Hansen, R., Albrechtsen, H. -J., Arvin, E. and Jørgensen, C. (2002b) Dynamics of biofilm formation in a model drinking water distribution system. *Journal of Water Supply: Research and Technology – AQUA* 51 (7), 399-406.

C) Boe-Hansen, R., Albrechtsen, H. -J., Arvin, E. and Jørgensen, C. (2002c) Bulk water phase and biofilm growth in drinking water at low nutrient conditions. *Water Research* 36, 4477-4486.

D) Boe-Hansen, R., Albrechtsen, H. -J., Arvin, E. and Jørgensen, C. (2002d) Substrate utilisation at low nutrients condition in a model drinking water distribution system. *Water Science and Technology: Water Supply* 2(4), 89-112.

In relation to the project two extended abstracts have been prepared for international conferences, these are however not included here.

Boe-Hansen, R., Albrechtsen, H. J., Arvin, E., and Jørgensen, C. (2000) Development of a model distribution system for studies of changes in water quality and microbial activity in drinking water distribution systems. 1st World Water Congress of the International Water Association, Paris, 3-7 July, 2000. CD-ROM.

Boe-Hansen, R., Martiny, A. C., Arvin, E., and Albrechtsen, H. J. (2002e) Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions. IWA Biofilm monitoring conference, Porto, Portugal, 17-20 March 2002.

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Rasmus Boe-Hansen

SUMMARY

The hygienic water quality of drinking water deteriorates during the transport between water works and consumer. The drinking water distribution network may be viewed as a large reactor where a number of chemical and microbiological processes are taking place. Especially, the microbial activity may cause numerous problems to the water supplies, and most of these are related to the indigenous biomass of the drinking water distribution network. Today, the routine quality monitoring is unable to diagnose the general hygienic quality of the drinking water, mainly due to the low frequency of the sampling and the use of inadequate microbial methods. However, there is presently a poor scientific basis to propose viable alternatives to the current practice.

The purpose of this study was to increase our understanding of the microbial growth dynamics in drinking water distribution networks. By establishing cause-relationships between bacterial growth and water quality, we may be able to construct computer models to predict water quality changes. These models can be applied in the future by water supplies to improve the overall hygienic quality of the drinking water. The studies of this project were especially related to Denmark where the drinking water generally contains a low level of nutrients and no disinfectants are added.

The dynamics of the microbial growth in drinking water networks is very complex, since a number of interacting processes are involved. Parameters like the hydraulic conditions, the amount of nutrients, the bioavailability of the nutrients, and the temperature are especially important. For the purpose of studying the processes, which affect the hygienic water quality, a model distribution system was constructed. The model system allowed for easy accesses to surface and water samples, while maintaining conditions realistic to distribution networks. A special emphasis was laid on constructing an experimental system able to mimic the hydraulic conditions commonly encountered in distribution networks.

The system was continuously fed drinking water containing a low level of assimilable organic carbon (AOC). The rate of biofilm formation was closely monitored during a period of 522 days by total bacterial counts (AODC), heterotrophic plate counts (HPC) and adenosine triphosphate (ATP) determination. The experimental data showed by modelling, that the growth rate of the bacteria was slow at 0.030 ± 0.002 d^{-1} during the biofilm formation phase. The quasi-stationary phase was reached after approximately 200 days, where the total bacterial counts was 2.6×10^6 cells/cm^2. The fraction of culturable cells (HPC/AODC) accounted for less

than 0.1% of the total bacterial counts. During the maturation of the biofilm, the microbial community changed properties in terms of specific ATP content and culturability.

A good correlation between bacterial growth and AOC removal was observed. The yield of the indigenous bacteria was 1.0×10^7 cells/μg ac-C and similar to literature values for pure culture bacteria isolated from drinking water. Mass-balances showed the cell production to be equivalent to an overall growth rate (biofilm + suspended bacteria) of 0.049 d^{-1}. The growth rate was significantly higher than the biofilm formation rate observed in the earlier study. This implied that the growth rate of the suspended bacteria in the water phase was considerable higher than the attached bacteria. Measurements showing that the water phase bacteria had a higher ATP content and faster leucine incorporation supported this hypothesis. Finally, water phase samples incubated without biofilm present showed that the suspended bacterial growth rate was approximately 10 times higher than the biofilm growth rate.

Environmentally realistic concentrations of benzoic acid were spiked to the drinking water of the model system in order to study the kinetics of the substrate utilisation. The use of radioactively labelled benzoic acid, allowed for tracking the carbon flow of the degradation during a short period of time. The degradation was successfully modelled using a no growth Monod expression. Results showed that only 2-4% of the carbon being degraded was actually incorporated into the biofilm. Nevertheless, the biofilm appeared to be responsible for the degradation of the benzoic acid added since no (or very little degradation) was observed in water samples incubated without biofilm. The results showed that the yield of the suspended bacteria was substantially higher than the biofilm bacteria.

The practical implication of these findings is that for large diameter pipes in non-chlorinated systems, the water phase bacterial growth may account for a major part of the cell production. The mature biofilm may act like a biological filter reducing the microbial aftergrowth, since biofilm bacteria (having a small yield) may compete for the substrate with water phase bacteria (having a higher yield). This could explain why elevated bacterial numbers are often observed in new drinking water installations, where a mature biofilm has yet to be established.

DANISH SUMMARY

Den hygiejniske kvalitet af drikkevand forringes som følge af transporten mellem vandværk og forbruger. Distributionssystemet kan opfattes som en stor bioreaktor, hvor mikrobielle og kemiske processer finder sted. Særligt den mikrobielle aktivitet kan give anledning til en række problemer, hvoraf de fleste kan relateres til den naturlige biomasse i ledningsnettet. Den almindelige drikkevandsovervågning kan ikke anvendes til karakterisering den generelle hygiejniske kvalitet af drikkevandet, primært, fordi der udtages for få prøver og fordi de mikrobielle metoder, der anvendes er uegnede til kvantificering af det naturlige samfund af mikroorganismer. Der er p.t. imidlertid ikke tilstrækkelig videnskabelig basis til at foreslå brugbare alternativer til den nuværende praksis.

Formålet med dette projekt er at øge forståelsen af den mikrobielle væksts dynamik i drikkevandsdistributionssystemer. Ved at etablere årsagsvirknings sammenhænge mellem bakteriel vækst og vandkvalitetsændringer, vil det i fremtiden være muligt at konstruere modeller, der tager højde for mikrobiologisk vækst i ledningsnettet. Modellerne, der kan anvendes af vandforsyningerne til at vurdere forskellige tiltag, der kan forbedre den hygiejniske kvalitet af drikkevandet. Dette projekts undersøgelser tager udgangspunkt i danske forhold, hvor drikkevandet er karakteriseret ved et generelt lavt indhold af næringsstoffer uden tilstedeværelse af desinfektionsmidler.

Dynamikken i den mikrobielle vækst i ledningsnettet er kompleks, fordi den påvirkes af adskillige indbyrdes afhængige processer. Følgende parametre har særlig stor betydning: De hydrauliske forhold, mængden af næringsstoffer, tilgængeligheden af næringsstofferne og temperaturen. Med henblik på at studere de mikrobielle processer, der påvirker den hygiejniske vandkvalitet, blev et modelledningsnet konstrueret. Modelledningsnettet betød overflade- og vandprøver let kunne udtages under forhold svarende til dem der eksisterer i ledningsnettet. Der blev lagt særlig vægt på, at model ledningsnettet var i stand til at efterligne de mest almindelige hydrauliske forhold i distributionssystemer.

Modelledningsnettet blev løbende tilført drikkevand med et lavt indhold af assimilerbart organisk kulstof (AOC). Biofilmdannelsen blev fulgt gennem 522 dage vha. totale bakterietællinger (AODC), kimtal (HPC) og måling adenosin trifosfat (ATP). Målingerne af biofilmdannelsen viste ved modellering, at bakterierne voksede langsomt med en hastighed omkring 0.030 ± 0.002 d^{-1}. Biofilmen nåede den kvasi-stationære fase efter ca. 200 dage, hvor det totale bakterietal var 2.6×10^6 celler/cm^2, heraf udgjorde andelen af dyrkbare bakterier (HPC/AODC) mindre end 0.1% af det

totale bakterietal. Bakterierne i biofilmens ændrede deres specifikke ATP indhold og dyrkbarhed efterhånden som biofilmen modnedes.

Der var en god sammenhæng mellem den observerede bakterievækst og AOC fjernelsen. Udbyttet for den generelle vækst var 1.0×10^{7} celler/µg ac-C, hvilket var i overensstemmelse med litteratur værdier for renkulturer af drikkevandsbakterier. Massebalancer viste, at produktionen af nye celler svarede til en overordnet vækshastighed (biofilm +suspenderede bakterier) på ca. 0.049 d^{-1}. Denne vækshastighed var signifikant højere end den tidligere observerede vækshastighed under biofilmdannelsen. Dette indikerede, at bakteriernes vækshastighed var væsentlig højere i suspension end i biofilmen. Hypotesen blev underbygget af, at de suspenderede bakterier havde et generelt højere ATP indhold og en hurtigere leucin indbygning. Inkubation af vandprøver uden tilstedeværelse af biofilm viste, at de suspenderede bakteriers vækshastighed var omkring 10 gange så hurtig som biofilmen.

Benzoat blev tilsat modelledningsnettet i miljørealistiske koncentrationer for at undersøge substratomsætningens kinetik. Ved brug af radioaktiv mærket benzoat kunne nedbrydningen følges nøje. Forsøgene viste at nedbrydningen kunne modelleres med et Monod udtryk uden mikrobiel vækst og at kun 2-4% af den nedbrudte benzoat blev indbygget i biofilmens bakterier. Ikke desto mindre syntes biofilmens bakterier at omsætte hovedparten af det tilsatte substrat, da nedbrydningen i vandprøver uden biofilm tilstede var meget lille. Resultaterne viste, at udbyttet for de suspenderede bakterier er meget større end for biofilmens bakterier.

Samlet viser resultaterne, at den suspenderede vækst kan udgøre en væsentlig del af den totale bakterieproduktion i drikkevandssystemer, dette gælder naturligvis især ved store rørdiametre. Den fuldt udviklede (modne) biofilm kan fungere som et biologisk filter, der reducerer den bakterielle eftervækst, fordi biofilm bakterier (der har et lille vækstudbytte) konkurrerer om substratet med suspenderede bakterier (der har et højt vækstudbytte). Dette kan forklare de høje bakterietal, der ofte forekommer i forbindelse med ibrugtagning af nye drikkevandsinstallationer, hvor en moden biofilm endnu ikke er etableret.

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ABBREVIATIONS

ac	Acetate
AO	Acridine orange, fluorescent stain used for total bacterial counts
AOC	Assimilable organic carbon
AODC	Acredine Orange direct count
ATP	Adenosine triphosphate
BDOC	Biodegradable dissolved organic carbon
C	Carbon
CFU	Colony forming unit
CSLM	Confocal scanning laser microscopy
CTC	5-cyano-2,3-ditolyl tetrazolium chloride, fluorescent stain used for viable bacterial counts
DAPI	4,6-diamidino-2-phenylindole, fluorescent stain used for total bacterial counts
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DVC	Direct viable counts
EPS	Extracellular polymeric substances
FDC	Frequency of dividing cells
FISH	Fluorescent *in situ* hybridisation
HPC	Heterotrophic plate count
Kings agar B	HPC media commonly used in routine monitoring of drinking water
MAR	Microautoradiography
MIC	Microbial induced corrosion
NOM	Natural organic matter
NOX	*Aquaspirillum* sp. strain NOX
P17	*Pseudomonas flourenscens* strain P17
PCA	Plate count agar, HPC media commonly used in routine monitoring of drinking water
PE	Polyethylene
PVC	Polyvinylchloride
R2A	HPC media developed for drinking water
Re	Reynolds number
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
TOC	Total organic carbon

1 INTRODUCTION

Microorganisms are always present in drinking water no matter how strict precautions are taken during the production and distribution. Drinking water being transported through distribution networks will be subject to both chemical and microbial quality changes.

Roughly, hygienic problems may originate from contamination by external microorganisms or growth of indigenous biomass. The activity of the indigenous microorganisms may cause numerous problems ranging from corrosion of pipes to human illness. The scale of these problems remains virtually unknown, since the monitoring frequency by the water supplies in the distribution networks generally is sparse and the microbial techniques used are inadequate for the characterisation of the indigenous microorganisms. Today, the small to medium size water supplies relies on only a few samples a year for their quality control. However, a rising demand from the consumers may eventually encourage the water supplies to introduce better quality control and quality assurance in the production of drinking water.

The task of efficiently monitoring the hygienic drinking water quality is large but not impossible. By gaining a better understanding of the cause-effect relationships controlling the microbial deterioration of the water quality and by describing the processes mathematically, we may be able to construct predictive models to help prevent and solve hygienic problems.

The purpose of the present project was to enhance the understanding of the growth dynamics of the indigenous microorganisms in drinking water distribution networks. A special attention was put on studying the microbiologically induced water quality changes. For the purpose of the planned studies an experimental distribution system was designed.

The thesis summarises the existing knowledge related to the experimental work performed. In the final chapter the major conclusions of the project is outlined. The reader is kindly referred to the papers of the appendices for a detailed description of the experimental work, which was carried out. Unless otherwise stated, this thesis concerns the distribution of cold drinking water originating from low nutrient groundwater.

2 DRINKING WATER PRODUCTION IN DENMARK

More than 99% of the drinking water in Denmark is produced from groundwater, which is unusual compared to other European countries where surface water normally constitutes a significant fraction of the abstracted raw water (Table 1).

Table 1. Sources of drinking water in selected European countries in 1997 (Spring water in brackets) (as cited by DVF, 2000).

Country	Groundwater (%)	Surface water (%)
Austria	49.4	0.8 (49.8)
Belgium	63.4	36.6
Denmark	99.6	0.4
Finland	57.7	42.3
France	59.5	40.5
Germany	64.9	27.9 (7.3)
Holland	62.6	37.4
Hungary	52.0	4.0 (44.1)
Italy	50.0	20.0 (30.0)
Romania	40.0	60.0
Slovakia	84.7	15.3
Spain	16.7	81.2 (2.1)
Sweden	23.9	76.1

The groundwater used for drinking water in most parts of Denmark is abstracted from deep aquifers, which means that the water is typically anaerobic and contains a low level of nutrients and microorganisms. The hygienic quality of the raw water is normally considered to be very good.

The structure of the Danish water supply is very decentralised, where 2851 common waterworks and up to 91.000 household wells are supplying the 5.3 million inhabitants of Denmark. The general tendency has been that the number of waterworks declines primarily due to emerging groundwater contaminants (pesticides and organic solvents) and economies of scale. As a result, the number of common waterworks has decreased by 27% during the recent two decades (DVF, 2000).

The drinking water treatment basically consists of two steps. Firstly an aeration, where methane and hydrogen sulphide are stripped from the water. The stripping reduces the aftergrowth potential by removing methane and sulphide, and furthermore oxygen is dissolved into the water. Secondly the sand-filtration, where the dissolved oxygen allows for chemical oxidation of iron(II) and biological oxidation of manganese(II) causing them to precipitate. The filters retain the precipitates, and biodegradable compounds like ammonium, traces of methane and hydrogen sulphide

are to some extent metabolised by the microorganisms in the filters. Disinfectants are rarely used, and if so only in relation to incidents were a microbial contamination has been inflicted upon the distribution network or when surface water is used in the drinking water production.

During the last decade, the water production in Denmark has decreased by 24% due to reduction of losses and a lower waste of water (DVF, 2000). Since the volume of the distribution network has remained virtually unchanged for this period of time, the production decrease has caused an equivalent increase in the average retention time. The increase may very well be larger, since the structure of the Danish water supply as previously described has been shifting towards a more centralised operation meaning that the distance between consumer and waterworks in average has increased. Longer retention times leads to a higher degree of deterioration of the water quality due to microbial activity. No statistical data concerning the changes in hygienic water quality during the period of declining water consumption are available, but temperature increases have been observed in several water distribution networks including the water supply of Copenhagen (DVF, 1998).

Most distribution networks in Denmark are constructed as interconnected loops. The primary purpose of this design is to increase the reliability of the system since the water can travel through alternative routes in case of pipe breaks. Secondary, the head-loss of the loop systems is generally reduced, which allows energy to be saved. The main problem of the loop systems is that the water flow is somewhat uncontrollable and unpredictable, hence the flow direction in the pipes may change during the day and the hydraulic retention time may be highly variable. When a local contamination of the drinking water occurs (i.e. by a back-suction incident), there is an urgent need to rapidly predict the spreading of the pollution within the network. Today, the water supplies are moving towards sectioning of their networks combined with transport modelling, which allows for a better control of the water flow, and retention time.

3 PROBLEMS CAUSED BY MICROORGANISMS

Most indigenous microorganisms in distributions systems are not directly harmful to humans, however they may cause numerous problems, which can be roughly divided into aesthetic, technical and health related problems.

Aesthetic problems are the most common cause of consumer complaints. The microbial activity can increase the turbidity of the water and cause bad taste and odour. Depletion of oxygen from the water may trigger the formation of sulphide, which causes a foul smell even at low concentrations.

Microorganisms may also cause a number of technical problems. The biofilm formation has been shown to increase the corrosion rate of the drinking water pipes (Lee et al., 1980). Microbial induced corrosion (MIC) is presumably costing water companies vast sums of money each year in maintenance cost. Insufficient stripping of methane can lead to severe aftergrowth in the sand-filters at the waterworks, which in extreme cases can increase the head-loss across the filter and even clog it. Biofilm formation will also increase the head-loss in the distribution system, due to the viscous nature of the biofilm (Stoodley et al 1998). When ammonia is present, concerning levels of nitrite may form as a result of incomplete nitrification. This may especially be a problem in mono-cloraminated networks since ammonia is usually added in order to increase the stability of the mono-cloramine (Lind, pers. comm.).

One of the greatest concerns in drinking water production is the risk of contaminating the drinking water with pathogenic microorganisms. Ingress of pathogens into the distribution system can rapidly lead to an infection of thousands of people. When pathogenic bacteria have entered a distribution network, the bacteria will be retained by the biofilm, in which they can remain for a prolonged period of time (Fass et al., 1996). In cold drinking water the role of the indigenous biofilm after a contamination by pathogenic organisms is disputable. The biofilm may act as a safe haven for the pathogenic bacteria where they are able to survive or maybe even grow (Keevil et al., 1995; Armon et al., 1997). In addition, the biofilm bacteria have been shown to be more resistant to disinfection (e.g. LeChevalier et al, 1988) and may therefore protect harmful organisms against disinfectants like chlorine. However, since most pathogenic bacteria are opportunists, the presence of a natural balanced biofilm adapted to the drinking water habitat may decrease the survival capability due to competition and grazing. As a result, the indigenous biomass can act as a barrier towards microbial contamination (Camper et al., 1985b; Sibille et al. 1998).

Some pathogens have a potential for growth in the distribution networks. The pathogenic *Legionella pneumophila* has been a serious problem in hot water installations all over the world. In a study by Szewzyk et al. (1994) a pathogenic *E.coli* was able to grow in a benzoic acid degrading pure culture biofilm and re-enter the water phase. Recent studies, have shown than *Helicobacter* sp. can be present in seemingly uncontaminated drinking water biofilms (Park et al., 2001), the genus include the species *Helicobacter pyleri* which is related to multiple diseases including one of the most common diseases in the western world – the gastric ulcer. Further research is needed to elucidate the problem of pathogenic growth in distribution networks.

4 DRINKING WATER MICROBIOLOGY

4.1 Properties

Microbial communities are characterised by their habitat. Danish drinking water is aerobic (contains oxygen) and oligotrophic (low nutrient conditions). The communities of oligotrophic environments are generally considered to be diverse with many different bacterial species present simultaneously, which is different from nutrient rich environments where fewer and more specialised bacteria tend to dominate. The oligotrophic bacteria are slow growing and appear to have lower growth efficiency compared to eutrophic systems (del Giorgio and Cole, 1998). Most bacteria observed in drinking water systems are heterotrophs, which means they obtain energy and carbon from degrading organic substances. Autotrophic bacteria like nitrifiers can also be present but usually in low numbers.

The aeration process increases the redox-potential as the environmental conditions are shifted from anaerobic to aerobic. This result in compounds previously being unavailable to the microorganisms now becomes available, as is the case of methane, ammonium, and hydrogensulphide. Generally, the organic substances are more easily degradable under aerobic conditions though there are examples of the opposite (e.g. some chlorinated compounds).

Ridgway and Olson (1981) were the first to show an extensive colonisation of bacteria on the inner surfaces of the drinking water distribution networks. The amount of bacteria present on the pipe walls varies since it depends on several factors. Table 2 lists the maximum number of bacteria observed by different studies in drinking water.

Table 2. Maximum total bacterial counts observed in drinking water biofilms.

Source	Max age (days)	Disinfectant present	Re*	Biofilm (cells/cm^2)	Reference
Surface water	35	Yes	22000	1.0-10.7×10^6	Clark et al. (1994)
Surface water	42	Yes	776	7-15×10^6	Fass et al. (1996)
Groundwater	70	No	-	0.7×10^6	Kalmbach (1998)
Not specified	90-150	No	6800	2.45×10^6	Ellis et al. (2000)
Surface water	167	Yes	117	4.9×10^6	Pedersen (1990)
Not specified	365	No	11080	1.9-3.7×10^4	Percival et al., (1998)
Groundwater	522	No	645	2.6×10^6	Boe-Hansen et al. (2002b)

*Reynolds number

The microbial community consists of a complex consortium of mainly bacteria, but protozoa, fungi and sponges have also been observed in the drinking water biofilm (Nagy and Olson, 1982; Sibille et al., 1998).

4.2 Biofilm morphology

The biofilm formed in drinking water systems consist of mainly bacteria and exopolymeric substances (EPS) excreted from the bacteria. The EPS enables a spatial organisation of the bacteria and may act as a barrier against environmental stress, such as starvation (Freeman and Lock, 1995) or disinfection (LeChevalier et al., 1988).

Previously, biofilm was considered to be a homogeneous layer covering the substratum, however introduction of confocal scanning laser microscopy (CSLM) has changed this perception. CSLM has two significant advantages compared to other forms of microscopy: a) it enables study of biofilm in its natural hydrated state, and b) it allows for observation of thin optical sections across the biofilm profile generating a three-dimensional image of the biofilm (Wimpenny and Colasanti, 1997). CSLM has revealed the structure to be highly heterogeneous (Stewart et al., 1995). The drinking water bacteria tend to form clusters (micro-colonies) held together by the EPS. The cell clusters has a mushroom structure with abundant channels, voids and pores (Costerton et al., 1994; Figure 1A) and stacks rising from the substratum (Keevil et al., 1995; Figure 1B). The open structure facilitates the transport of molecules and even particles from the bulk water phase into the biofilm (Stoodley et al., 1994; de Beer and Stoodley, 1995). The heterogeneity of the biofilm permits formation of different microenvironments and thereby different ecological niches inhabited by specialised microorganisms.

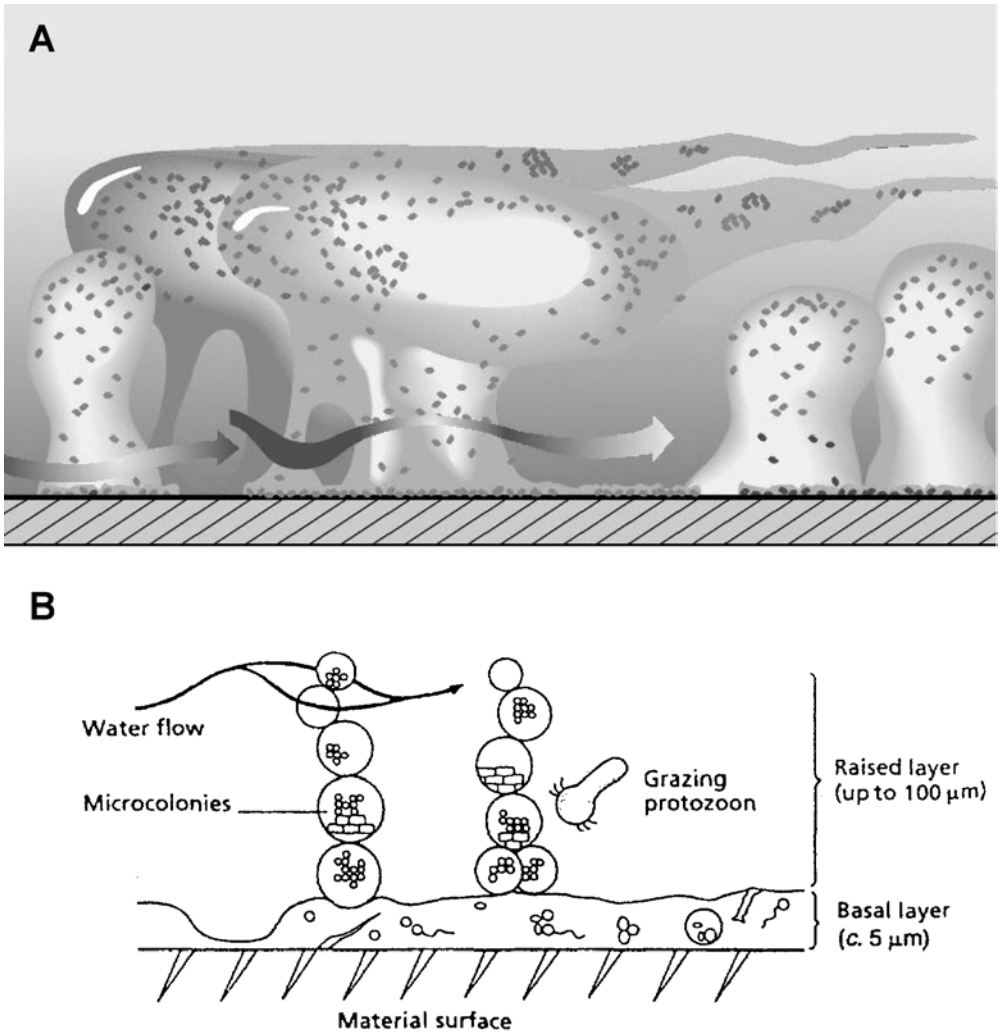

Figure 1. Two conceptual biofilm models. A) Costerton et al., 1994. B) Keevil et al., 1995

Generally, the structure of the biofilm seems to be dependent of two main factors: the load of nutrients and the rate of detachment (Wimpenny and Colasanti, 1997; Van Loosdrecht et al., 1997; Hermanowicz, 1998). At low nutrient conditions, the nutrients may be depleted in a zone around a bacterial cluster prohibiting the bacteria in colonising the area. The phenomenon is well known from colonial growth on agar plates. When flow rates are high the bacteria tend to form a patchy but smooth biofilm, whereas the biofilm formed at low flow rates is heterogeneous with many pores (Van Loosdrecht et al., 1995). The reason for this seem to be a combination of the higher shear forces leading to a higher rate of detachment and the greater flux of nutrients into the biofilm.

4.3 Microbial activity

Large pipe diameters favour the influence of suspended bacteria while small diameters favours biofilm. If a biofilm density of 2.6×10^6 cells/cm^2 and a suspended concentration of 1.1×10^5 cells/mL is considered (Boe-Hansen et al., 2002b); the suspended bacteria constitutes 17% of the total biomass in case of a 200 mm diameter pipe and only 5% for a 50 mm diameter pipe. The small pipe diameters are found in the house installations, where the water may be stagnant for a long period, hence the most significant water deterioration may occur within the plumbing of the houses.

The relation between suspended bacteria and the biofilm has been subject to several studies. The properties of the suspended bacteria are significantly different from the biofilm as large metabolic differences can occur (Bonin et al., 2001). This has been observed in non-chlorinated drinking water in a study by Boe-Hansen et al., (2002c) where the activity of the suspended bacteria was remarkably higher than the biofilm bacteria (Table 4).

Table 4. Metabolic differences between water phase and biofilm bacteria. Specific activity of the bacteria compared to total bacterial counts (Boe-Hansen et al., 2002c).

	Water phase	Biofilm
Culturability, 14 days (CFU/cell)	0.011-0.029	0.0005
Specific ATP content (10^{-15} g/cell)	3.1-6.7	1.1
Specific leucine incorporation (10^{-21} mole/h/cell)	18.7-47.9	2.2

Van der Wende et al. (1989) concluded that detachment of biofilm bacteria accounted for the increase in HPC observed in the bulk phase, while growth of suspended bacteria was negligible. However, the results of this study are not unambiguous, since the results can be explained by a reduction in culturability as observed by Boe-Hansen et al. (2002c). Observations by Manz et al. (1993) indicated the biofilm bacteria to have a higher specific content of ribosomal RNA, which is usually equivalent to a higher activity. The drinking water of this study had a residual concentration of chloramine, which generally favours biofilm growth. Investigations by Boe-Hansen et al. (2002c) showed that bulk water phase growth accounted for a significant part of the total cell production in a non-chlorinated model distribution system. The same study showed the net growth rate of the suspended bacteria to be more than 10 times as high as the biofilm.

The present knowledge of yield coefficients of drinking water bacteria is limited, especially at environmentally realistic nutrient concentrations. Yield coefficients for different carbon sources in drinking water are listed in Table 5.

Table 5. Observed yield coefficients of drinking water biofilms.

Substrate	Concentration (µg C/L)	Yield (g C/g C)	Reference
Benzoic acid	2	0.024	Boe-Hansen et al. (2002d)
	10	0.034	
Amino acids	500	0.12	Ellis et al. (2000)
	1000	0.080	
	2000	0.084	
Carbohydrates	500	0.089	Ellis et al. (2000)
	1000	0.038	
	2000	0.046	
Humics	500	0.045	Ellis et al. (2000)
	1000	0.034	
	2000	0.035	

The yield of the biofilm growth in drinking water is low compared to other microbial environments (Button, 1985). The low yield might suggest that the bacteria are using energy for cell maintenance rather than growth, which can be a good survival strategy in a low nutrient environment. The yield coefficient is obviously dependent on the composition and concentration of the substrate used for the bacterial growth (Ellis et al., 2000).

5 MICROBIAL CHARACTERISATION METHODS

In this chapter some of the microbial characterisation methods, which have been applied to drinking water is described and discussed.

5.1 Microscopic techniques

Visualisation of organisms by microscopic techniques is the basis of microbiology. The application is widespread and the individual methods are very diverse ranging from phase contrast to electron microscopy. Environmental samples are usually stained by fluorochromes to facilitate the examination by epi-fluorescent microscopy.

Total microbial counts are included in virtually every microscopic study. Two nucleotide stains that produce similar results are prevalent: Acridine orange (AO) and 4,6-diamidino-2-phenylindole (DAPI). Both stains are easy available and very reliable. In the environment, a majority of the bacterial cells present are usually non-culturable by conventional techniques (e.g. plate counts).

To detect viable bacteria without cultivation the direct viable counts (DVC) and the CTC method have been proposed.

The DVC is based on inhibiting the bacterial DNA replication using an antibiotic (nalidixic acid), while other metabolic activities remain unaffected (Kogure et al., 1979). If appropriate nutrients are available and the bacteria are sensitive to the antibiotic, the cells will elongate during incubation. Several studies have applied the DVC technique to drinking water communities (Byrd et al., 1991; Kalmbach et al., 1997b). The method is relatively tedious since it involves a size determination of the cells, though automated image-analysis has improved the method.

Physiological stains that indicate metabolic activity in the bacteria have been applied to some extent. Yu and McFeters (1994) reported 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to be an effective indicator for metabolic active bacteria in drinking water biofilms. However, recent studies have shown that CTC has a toxic effect on bacterial cells by inhibiting protein synthesis (Ullrich et al., 1996; Sevais et al., 2001). The later study indicated that the CTC active cells accounted for less than 60% of the total active bacterial production. Observations by Boe-Hansen et al. (unpublished data) showed an activity of a drinking water biofilm below the sensitivity of CTC method.

Recently, the most important recent advance in environmental microbiology is the introduction of molecular techniques for the identification of bacteria in their natural

habitat. The most commonly used technique is fluorescent *in situ* hybridisation (FISH), where a sequence of nucleotides labelled with a fluorescent marker is introduced into the cell as a probe. When the probe meets a complementary sequence on the bacteria's rRNA, it reversibly binds to it, subsequently allowing for microscopic detection. Since the bacterial rRNA is unique on a strain level, application of appropriate probes can be used in the characterisation and tracking of bacteria on different phylogenetic levels. An example of two levels of FISH applied to a drinking water biofilm is shown in Figure 2.

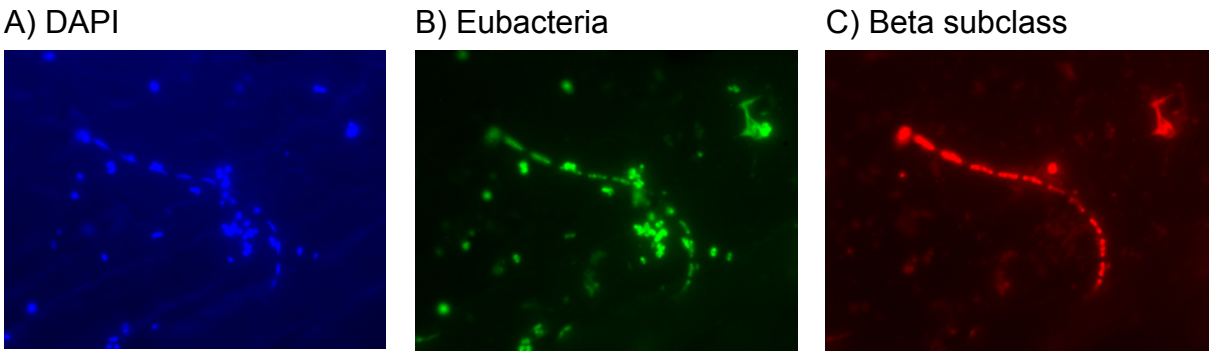

Figure 2. Two levels of FISH applied to drinking water biofilm (Riemann and Boe-Hansen, unpublished data). A) Total bacterial stain (DAPI). B) Eubacterial probe. C) Probe targeting the beta subclass of the Proteobacteria.

The use of FISH potentially allow the microbiologist to study bacteria without culturing (review by Amann et al., 1995). However, applying FISH to the bacterial community of low nutrient drinking water poses some difficulties, since bacteria of oligotrophic systems generally has a low content of ribosomes (due to their slow growing nature). This means that the probe will only bind to a few sites within the bacteria and consequently the fluorescent signal will be proportionally weak. There are several possible ways to by-pass this problem (Amann et al., 1995; Kalmbach 1998; DeLong et al., 1999), but the methods are not readily applicable for routine measurement.

5.2 ATP

ATP (adenosine triphosphate) is present in all organisms as an energy carrier molecule. ATP can be measured rapidly with great sensitivity using the light emitting luceferine-luceferase enzyme reaction. The ATP assay can be used to estimate microbial biomass, though not without difficulties, since cells may alter their ATP content depending on their nutritional state e.g. the ATP content has been shown to decline during starvation (Hamilton and Holm-Hansen, 1967). Information about the

nutritional state of a population can be obtained by comparing the ATP amount with the total bacterial counts.

Van der Kooij et al (1995) and Boe-Hansen (2002b) have used ATP measurement as a measure for total biomass in drinking water systems. The short time of analysis, a simple measurement procedure and the low operation cost makes the ATP assay perhaps the most promising technique for the water supplies routine monitoring of the general hygienic quality of drinking water.

5.3 Plate counts

Heterotrophic plate counts (HPC) tests the bacteria's ability to form visible colonies under specified conditions. The HPC method is by far the most common and widespread procedure for water supplies to monitor hygienic water quality. The method is generally easy to use but reflects only the natural population to a limited degree. The choice of media, incubation temperature and time for the culturing of the bacteria is of major importance. Ideally, the media and temperature should mimic the natural habitat of the bacteria, but the use of a standard procedure is preferable to allow comparisons between different systems. PCA, Kings agar B, and R2A are the most commonly used media, where the R2A media (Reasoner and Geldreich, 1985) is generally considered to have the highest sensitivity towards the indigenous microbial community of drinking water (Figure 3).

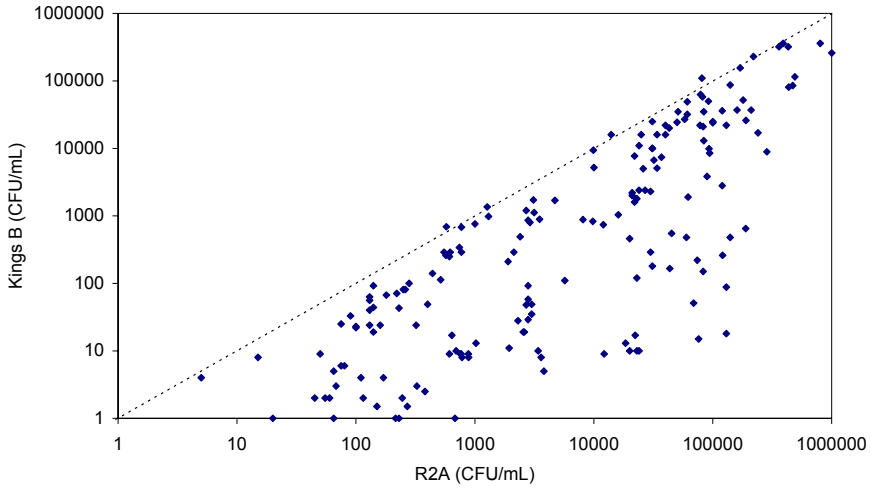

Figure 3. Comparison between HPC on Kings agar B and R2A media. Samples collected from 10 different Danish water supplies (Danish EPA, unpublished).

Heterotrophic plate counts have been applied extensively in a large number of studies to quantify the general microbial activity. The method however, seems unfit for

studies of bacteria in drinking water, since it is only able to detect major changes in the bacterial biomass, due to large measurement variations (Boe-Hansen et al. 2002c). As a monitoring technique the most severe flaw of the HPC is that the method is extremely slow, as it takes several days to get a result.

5.4 Activity measurements

Measurement of incorporation rate of radioactive labelled amino acids is an indirect measure of the bacterial activity, which is related to the bacterial growth rate (e.g. Riemann et al., 1990). The principle of the method is to measure the bacterial uptake during a period of time by addition of a known concentration of amino acid to the external environment. Several different amino acids have been applied, but thymidine and leucine are generally considered to be the most suitable for bacterial production studies in aquatic systems (Riemann and Bell, 1990). The studies can be done at high sensitivity and accuracy, since radioactively labelled compounds can be used easily. Biomass re-suspension procedures may be performed harsher because the measurement does not require the cells to be intact like in microscopic studies.

Bacteria are able to take up thymidine present in the external environment and incorporate it into their DNA. Furhman and Azam (1980) who originally introduced measurement of the bacterial thymidine incorporation observed that the incorporation was closely linked to the production of new cells. A disadvantage of the method is that bacteria can be actively respiring without growing (i.e. synthesising DNA), this may very well be the prevalent situation in oligotrophic environments. The bacteria may further complicate the data interpretation by converting and incorporating thymidine into protein (Riemann and Bell, 1990). Finally, the bacterial *de novo* synthesis of thymine is not completely suppressed, which allow for some degree of isotope dilution to occur.

Alternatively, leucine has been applied, since it is exclusively incorporated into the protein of the cell, where it constitutes approximately 7% of the total bacterial protein (Simon and Azam, 1989). A large fraction (>50%) of bacteria in marine aquatic systems have been shown to be able to take up external leucine, and >90% the leucine was incorporated into protein (Kirchman et al., 1985). Again, the main weakness of the method is that the bacterial *de novo* synthesis of leucine is not completely suppressed, in spite of the presence of leucine in the environment (Simon and Azam, 1989). This means that the radioactive leucine may be internally diluted within the cell. However, the leucineincorporation method produces at least an underestimate of the bacterial production (Kirchman et al., 1986).

Studies of specific cell uptake of radioactive labelled compounds have been successfully combined with microscopic studies in microautoradiography (MAR) (e.g. Bright and Fletcher, 1983; Lee et al., 1999). A microscopic slide is covered with a nuclear track emulsion, which in effect reveals single bacteria that has taken up radioactivity. The method has a great potential for studies of microbial substrate utilisation, though it is currently considered difficult to perform (Nielsen, pers.comm.). The MAR technique have been applied in marine and wastewater samples, but has yet to be applied in studies of drinking water biofilms.

5.5 Substrate

The composition of the substrate in drinking water is a complex mixture of different compounds dependent of the origin of the water. The microbial growth are usually limited by the content of microbial available organic matter, which normally only constitutes a small fraction of the total amount of organic carbon (TOC). Analytical characterisation methods are not applicable in practice for determination of microbial available substrate, which is why bioassays are widely used. The two most common methods are the determination of biodegradable dissolved organic carbon (BDOC) and assimilable organic carbon (AOC).

The BDOC assay is based on measuring the reduction of dissolved organic carbon (DOC) as a result of complete biological degradation (Servais et al., 1987). A water sample is inoculated with a microbial community of same origin as the samples, which leads to a microbial consumption of substrate. After an incubation period (normally 3 or 5 days) the amount of DOC is measured and compared to the original amount. For drinking water samples, the inoculum may be sand from the filters at the water treatment plant (Joret et al., 1991). The method has proven useful for studies of microbial available substrate in distribution networks (Servais et al., 1995). The detection limit for BDOC is determined by the detection limit of the TOC measurement, which is usually 0.1 mg/L or higher.

An alternative method is the determination of assimilable organic carbon (AOC) proposed by Van der Kooij et al. (1982), where a pasteurised water sample is inoculated with pure bacterial cultures. The growth of the bacteria is monitored by HPC until a maximum cell number is reached. The maximum cell number is compared to growth of the pure cultures on a known carbon source (e.g. acetate). The analysis is normally carried out using the two bacteria *Pseudomonas flourenscens* strain P17 and *Aquaspirillum* sp. strain NOX simultaneously. A typical growth curve for the AOC determination in Danish drinking water is depicted in Figure 4.

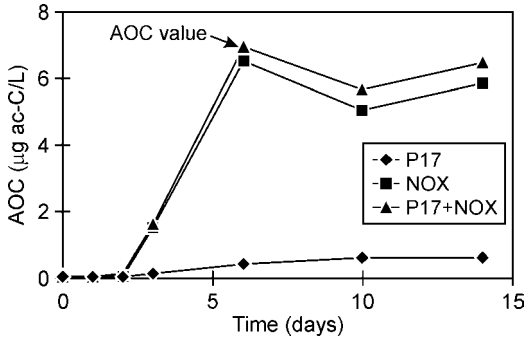

Figure 4. Growth curve for determination of assimilable organic carbon (AOC) in a Danish drinking water sample (the cell concentrations has been divided by the acetate yield coefficients for the two bacteria respectively) (Boe-Hansen, unpublished).

Each of the two methods has advantages compared to the other. The AOC method is more standardised than the BDOC and the detection limit for the method is much lower. However, the BDOC measurement is closer to the "real-life" situation, primarily due to the fact that the degradation potential of a few AOC test bacteria is more limited than an indigenous bacterial community.

It should be emphasised that the two methods are fundamentally different, the AOC method relates directly to the growth of bacteria, while the BDOC relates to the utilisation of organic substrate. No linear correlation can be expected between the two methods (Woolschlager and Rittmann, 1995), which is why they ideally both should be used.

For the purpose of studying drinking water containing a low level of biodegradable organic matter (<0.2 mg/L), the AOC method is generally considered to be more reliable than BDOC, due to the lower level of detection (Huck, 1990).

5.6 General

Microbial measurements in drinking water poses special difficulties, due to the low amount of bacteria present and their low activity, hence the techniques best suitable are often the most sensitive available. Selection of the proper microbial characterisation method is very important since the type of information obtained depends on the method used. However, no single method can be recommended for microbial studies in drinking water, which is why combinations of different methods should be used.

Apart from the microbial methods, the sampling procedure of the biofilm is crucial. Few methods are applicable directly to the biofilm hence the attached bacteria usually have to be removed from the substratum. Removal of intact cells may be a difficult

task, since the choice of procedure is a balance between obtaining high removal efficiency and minimising the stress, inactivation or even destruction of the cells. Possible procedures include flushing (LeChevallier et al., 1987), sonication (Clark et al., 1994), centrifugation (Camper et al., 1985a), and swapping (Boe-Hansen et al., 2002b) which can be combined with the use of detergents.

6 EXPERIMENTAL SYSTEMS

In the following chapter different experimental systems for studies of the microbiology of drinking water systems is discussed.

6.1 Environmental sampling

When a pipe sample is recovered, one will often find the inner surfaces to be covered with encrustations. The amount and type of encrustation is dependent of the water type and age of the pipe. The durability of drinking water pipes is commonly assessed to be 30-100 years, which allow for formation of thick encrustations. In Denmark a rough inner surface of iron and calcium precipitates are very abundant (Figure 5).

Figure 5. Encrustations in a typical drinking water pipe.

The precipitates may be involved in the formation of anaerobic zones, which allows for proliferation of anoxic bacteria e.g. sulphate reducers (Tuovienen and Hsu, 1982). The rough surface impedes direct microscopy of the biofilm so a re-suspension of the biofilm may be needed.

There are several problems in examining natural biofilm from distribution networks. The sampling is generally difficult, since the pipes can be inaccessible (buried in the ground). Undisturbed biofilm samples can be hard to obtain when the pipe sections have to be dismantled. The experiments cannot be repeated since the sampling is destructive and the sampled pipe sections will have to be replaced by new pipes that may alter the entire system. Nonetheless, several studies have investigated samples taken directly from drinking water systems (e.g. Allen et al., 1980; Ridgway and Olson, 1981; LeChevalier et al., 1987; Zacheus et al., 2001).

6.2 Culturing of biofilm

To allow for easier sampling and more controlled experimental conditions, insertions of test surfaces into water systems has been used in several studies. In theory, the approach is simple; test surfaces are being placed within a flow of drinking water and subsequently harvested after an appropriate amount of time.

Donlan and Pipes (1988) used a sampling device, which allowed test surfaces to be placed in the middle of the water flow. However, their approach introduces local differences in shear stress that may influence the biofilm formation. From a hydraulic point-of-view it is better to have the test surface even with the inner surface of the pipe (Bagh et al., 1999).

Probably the most popular way to culture drinking water biofilms is the Robbins device (McCoy et al., 1981), where a microscopic slide or a similar surface is exposed to a constant flow of water (Figure 6a). The biofilm formed is very well suited for *in situ* microscopic studies. The Robbins device is cheap, easy to use and very reliable, though the hydraulic conditions under which the biofilm forms are not well defined and practically uncontrollable. The set-up is especially unfit for studies of water quality since the retention time is interlocked with the water velocity.

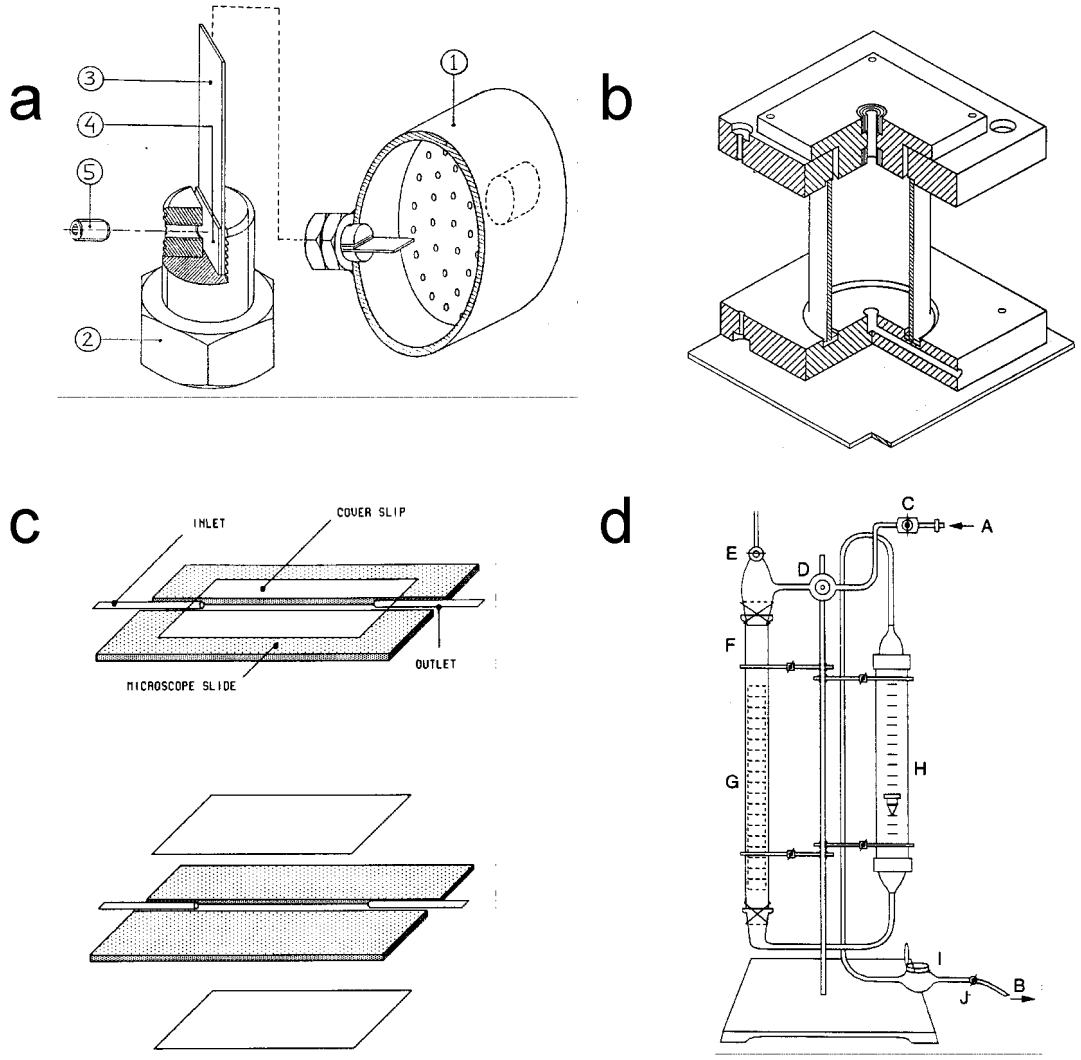

Figure 6. a) The Robbins device (Kalmbach et al. 1997a). b) The RotoTorque (Van der Wende et al., 1988). c) Flow cell (Caldwell and Lawrence, 1988). d) The biofilm monitor (Van der Kooij et al., 1995).

The RotoTorque system (or the annular reactor) was developed in order to provide well-defined hydraulic conditions at the test surfaces. The system consists of a continues flow stirred reactor with a spinning drum (Figure 6b). By changing the rotational speed, the hydraulic conditions can be changed in order to mimic different flow conditions. The system is not well suited for studies of water quality changes, since surfaces at top and bottom are exposed to poorly defined hydraulic conditions. The biofilm formation at these surfaces will be formed under hydraulic conditions less controllable than at the test surfaces. Nevertheless, series of RotoTorques have been applied in studying water quality changes caused by drinking water biofilm (Van der Wende et al., 1989). A number of variations of the design exist and some are commercially available.

A simple way to culture biofilm in the laboratory is flow cells. The flow cells allow the laboratory to growth biofilm under relatively controlled conditions. A flow of water is led through removable parallel slides on which the biofilm are forming (Figure 6c). By changing the water flow, the hydraulic conditions can be varied. The method is unsuitable if a large number of replicates are needed because it can be difficult to provide the exact same conditions. The main advantage of the method is that it allows the study of biofilm in its hydrated state, since the flow-through conditions can be maintained while non-destructive direct microscopy is performed.

The biofilm monitor, developed by Van der Kooij et al. (1995) enables a large number of surface samples to be taken (40 per device). Small cylinders are placed within a glass column in a flow of water. Both the inner and outer surface of the cylinders is in contact with the water (Figure 6d). The high number of samples allows for replicate measurement in detailed studies biofilm formation kinetics. The main concern is that the hydraulic conditions at the surfaces of the cylinders are inhomogeneous, which introduces additional variance.

6.3 Model distribution systems

The purpose of model distribution systems is to closely mimic the conditions of real life drinking water networks. Model distribution systems are especially useful in determining water phase biofilm interactions since the hydraulics and the surface to volume ratio can be similar to drinking water networks. Generally two types of model distribution systems exist: the one-pass system and the recirculation systems. One-pass systems have been applied in a few studies (LeChevalier et al., 1990; McMath et al., 1999), but it is rarely the natural choice. The main disadvantage of the one-pass system is that the water velocity determines the retention time, thus if realistic levels are to be attained very long pipe lengths are needed. The largest model distribution system yet constructed has a length of 1.3 km(!) (McMath et al., 1999), which is still only a fraction of the length of a small distribution system.

The obvious way to by-pass the need for long pipe lengths is to recycle the water and thus enable independent control of water velocity and retention time (Figure 7).

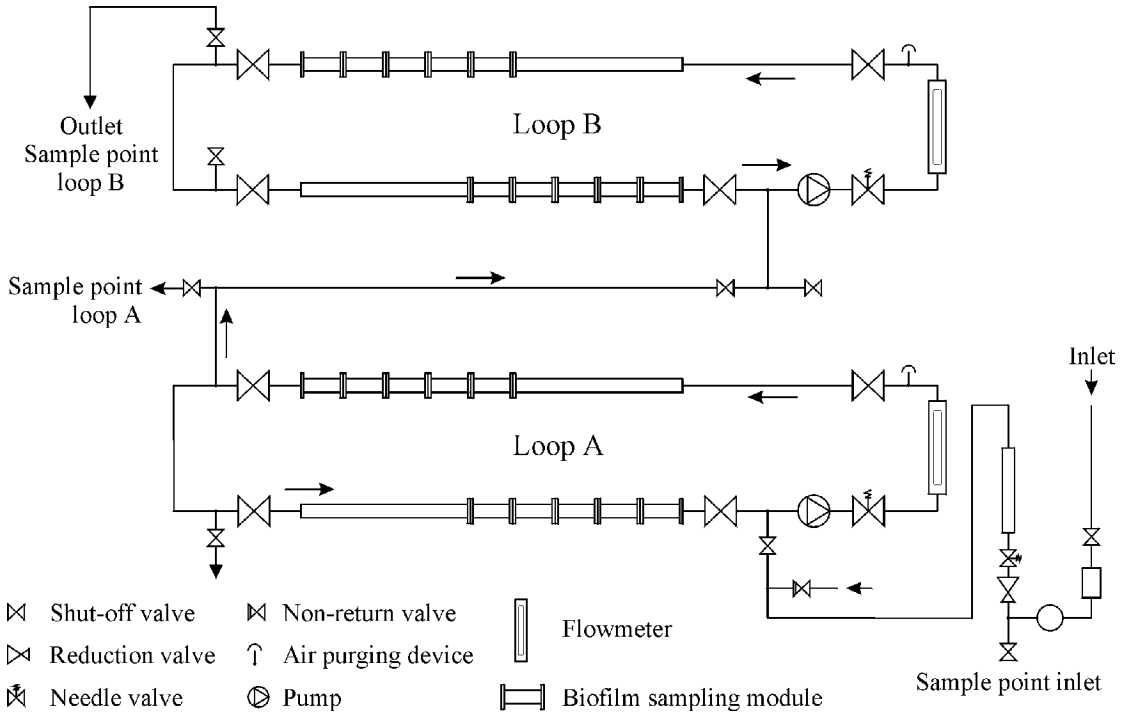

Figure 7. Recycled model distribution system (Boe-Hansen, et al. 2002a).

Model distribution systems have been applied by Lee et al. (1980), Mathieu et al (1993), Piriou and Levi (1994) and Boe-Hansen et al. (2002a). Contrary to the one-pass system, horizontal gradients in the nutrient concentration are not present in the recycled system hence the biofilm is exposed to the same conditions throughout the loop. Different nutritional states of the biofilm can be studied simultaneously by constructing several loops in series.

The construction of model distribution system should relate to a number of requirements listed in Table 5.

Table 5. Design requirements for model distribution systems (Boe-Hansen et al., 2002a).

Primary requirements	Access to biofilm and water samples
	Realistic hydraulic conditions
	Constant temperature
	Construction of inert materials
Secondary requirements	Independent control of flow rate and retention time
	Vast number of biofilm samples
	Connection to municipal distribution system
	Closed system
	Sampling of plane surfaces

The fulfilment of the design criteria is obviously dependent on the type of experiments to be performed.

6.4 Choice of experimental system

As previously mentioned, a number of considerations should be included, when choosing an experimental system. Naturally the choice of system should reflect the type of study to be performed. Table 6 summarises the advantages of the different experimental systems.

Table 6. Advantages of different experimental set-ups.

	Robbins device	Flow cells	Roto-Torque	Biofilm monitor	Model system
Well-defined hydraulics	÷	++	++	÷	++
Controllable flow rate	÷	++	++	÷	++
Amount of samples	+	÷	+	++	+
In situ microscopy	++	++	++	÷	++
Water quality studies*	÷	÷	÷	÷	++
Low construction costs	++	++	+	++	÷

*For water quality studies the following requirements must be met: a) The surface to volume ratio should be realistic to drinking water networks. b) The biofilm of the set-up must be homogeneously exposed to the same hydraulic conditions. c) The retention time should be independently controlled.

If properly constructed, the model system unites the advantages of the different biofilm culturing systems.

7 FACTORS AFFECTING WATER QUALITY

The main factors affecting water quality in drinking water networks are:

- The composition of the microbial community
- The amount and bio-availability of substrate
- The hydraulic conditions
- The temperature
- The presence of inhibitory compounds
- The properties of the substratum

This chapter gives a qualitative description of these factors controlling water quality changes in distribution systems.

7.1 Composition of the microbial community

One of the most important factors determining the effect on water quality of bacterial activity is traditionally omitted from the models, namely the composition and properties of the microbial community present. Differences in community composition may explain a large part of the variation, which is encountered between different sampling sites. However, our knowledge of the type of microorganisms actually present in drinking water and their properties is very limited, due to the low culturability of the bacteria indigenous to drinking water. Studies using the classical culturing approach have frequently found different genera of *Pseudomonas* to be predominant (e.g. LeChevalier et al., 1987). Kalmbach et al. (2000) applied the FISH technique in showing that the *Aquabacterium commune* was dominant in three different water distribution systems though a similar study by Martiny (2000) was unable to support these findings in the model distribution system used by Boe-Hansen et al. (2002a). The non-culturing characterisation methods have been tremendously improved during the last decade, however none have reached the stage where they can be implemented into predictive models of water quality changes in distribution systems. The dynamics of the selectivity of microorganisms at the surfaces appear to be extremely complex as it involves a great degree of interaction between microorganisms, such as cell-to-cell signalling (Davies et al., 1998), competition for nutrients (Zhang et al., 1995), and co-operative degradation (Van Ginkel, 1996).

7.2 Amount and bio-availability of substrate

The aeration of the drinking water, at the waterworks results in compounds being unavailable under the anaerobic conditions of the groundwater aquifer becomes available to the microorganisms. The energy obtained by the bacteria when degrading organic substances under aerobic conditions is higher than for anaerobic growth, due to the greater change in Gibbs free energy (Stumm and Morgan, 1995). This means that the bacterial yield factor is generally higher, which in effect enhances the microbial growth potential.

The microbial growth in drinking water is usually limited by the amount of microbial available carbon (Van der Kooij, 1992), though phosphorus in some cases has been shown growth limiting (Miettinen et al., 1997; Sathasivan and Ohgaki, 1999). A study by Boe-Hansen (2002d) showed that the bacterial growth was increased when carbon was added in low concentration indicating that organic carbon was growth limiting while no effect of adding phosphate to AOC samples was observed (Boe-Hansen, unpublished). The amount of microbial available carbon has been observed declining, as the water is transported through the distribution network (LeChevalier et al., 1987; Van der Kooij, 1992). This has been confirmed by investigations in model distribution systems (Block et al., 1994; Boe-Hansen et al., 2002d) (Figure 8).

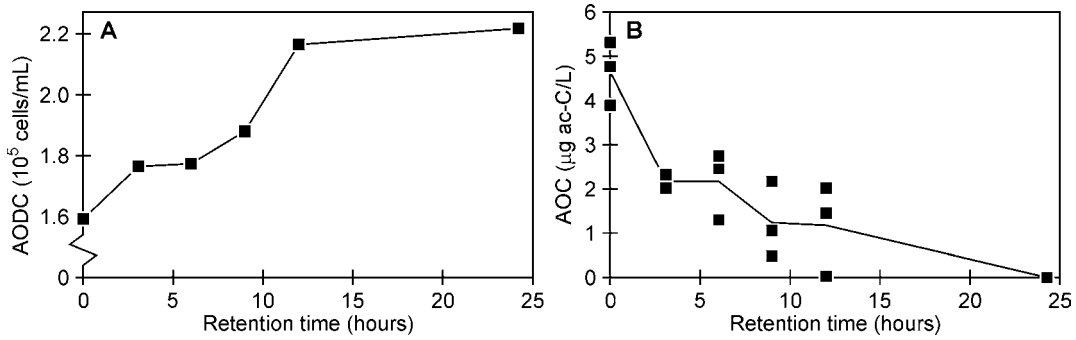

Figure 8. Water quality changes as a function of retention time in a model distribution system (Boe-Hansen et al., 2002d). A) Total bacterial counts in bulk water phase. B) AOC concentration.

Even low levels of organic carbon can support microbial growth in drinking water. Van der Kooij (1992) proposed that water containing an AOC level of 10 µg ac-C/L or lower could be considered biological stable, while LeChevalier et al. (1991) proposed 50 µg ac-C/L as a limit for coliform regrowth. Servais et al. (1995) suggested that water containing a BDOC level below 0.15 mg/L could be considered biologically stable. Though these values may be applicable as a rule-of-thumb for practical purposes, the scientific basis for proposing them is poor and considerable biological growth has been observed at AOC concentrations less than 10 µg-ac/L (Boe-Hansen et al., 2002c).

Charged and hydrophobic compounds will to a certain degree adsorb to the surfaces of the system. The attached bacteria may profit by an elevated level of nutrients present at the surfaces compared to the suspension, though the exact effect remains non-quantified.

7.3 Hydraulics

The physical construction and the rate of water consumption determine the hydraulic conditions in the drinking water network. The consumption is highly variable during the day and as a consequence the age of the water in one location will change.

Deterioration of the hygienic water quality as a function of retention time has been observed in model distribution systems as seen in Figure 8a. Since most distribution systems are designed with the primary goal to ensure a steady supply of water, the pipes tend to be over-dimensioned to avoid capacity problems. However, large pipe diameters equals low water velocity and thus longer retention time in the distribution system. Thus preventing transport capacity problems may introduce new problem like reduction of the hygienic water quality.

The Reynolds number is commonly used to describe the level of turbulence of the pipes. It has been shown empirically that flow with a Reynolds number (Re) less than 580 are laminar and at Re > 750 the flow is practically always turbulent (Engelund and Pedersen, 1978). The flow in the water pipes is shifting between the laminar and the turbulent flow regime during the day. A computer simulation (Aquis, Water Tech) of the distribution of Reynolds numbers in a typical Danish municipal network during an average day is depicted in Figure 9. The Figure shows that the flow in this particular network could be considered non-turbulent (Re < 750) 61% of the day.

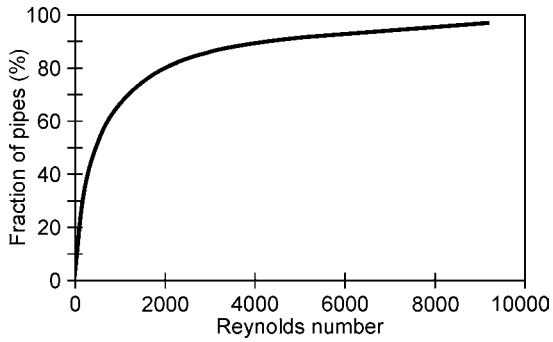

Figure 9. Distribution of Reynolds numbers in a distribution system during an average day (Boe-Hansen et al., 2002a).

Even if the flow is mostly laminar, short periods of high flow may have a profound effect on the biomass of the distribution system, since the detachment rate of microorganisms is increased during these periods of high turbulence.

7.4 Inhibitory compounds

Presence of inhibitory compounds reduces the microbial activity. A large spectrum of different compounds have inhibitory effects towards bacteria ranging from biocides such as chlorine added to the drinking water to anti-microbial components secreted by bacteria (Bernet-Camard et al., 1997). The biofilm bacteria have been shown to be less susceptible to inhibitory compounds than bacteria in suspension (Figure 10).

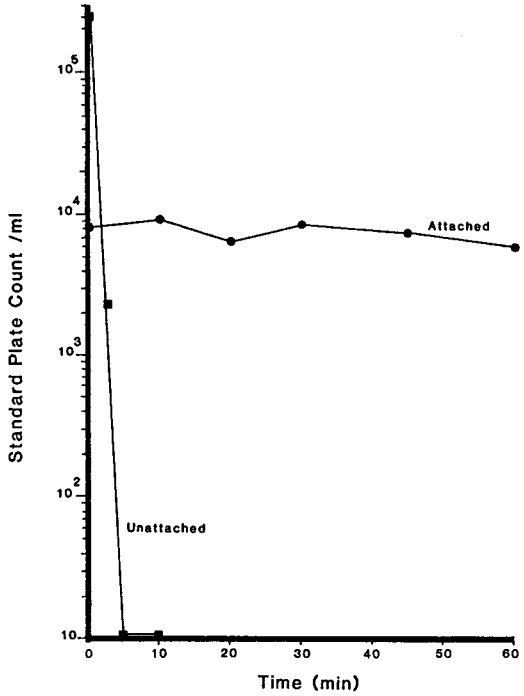

Figure 10. Survival of natural occurring bacteria exposed to 2 mg free chlorine (LeChevallier et al., 1984)

The cause of the resistance has been subject for discussions. In the past, the prevalent theory was that the lower disinfectant efficiency was caused by the disinfectant needing to penetrate the biofilm by diffusion, and thus the biofilm bacteria was exposed to a lower concentration of the disinfectant. However, studies have shown that the EPS of the biofilm may be involved in a more active inactivation of inhibitory compounds (Stewart et al., 1996).

Another commonly encountered inhibitor of biofilm formation in drinking water systems is ions released from copper pipes.

7.5 Temperature

For groundwater based drinking water production, the temperature is usually close to the yearly average temperature, which is 9-12°C in Denmark. A positive correlation between temperature and biofilm formation has been observed by Donlan et al. (1994). Water supplies generally tend to keep the temperature as low as possible in order to minimise the bacterial aftergrowth. In Danish distribution networks, high temperatures are often encountered as a result of high retention times.

Keeping the temperature low reduces the risk for pathogenic proliferation and survival since the optimal temperature for most pathogens is close to the human body temperature.

7.6 The substratum

The effect of the substratum on biofilm formation has been subject to a number of studies (Pedersen, 1990; Clark et al., 1994; Van der Kooij et al. 1995; Percival et al., 1998; Niquette et al., 2000). When compared to an inert surface like glass, PE and PVC surfaces have been shown to enhance the biofilm formation (Rogers et al., 1994), while copper surfaces inhibited the formation (Rogers et al., 1994). A recent study showed inhibition from molybdenum, which is known to be leaking from stainless steel grade 316 (Percival, 1999). It is however questionable whether these effects will persist after the surfaces has been covered by a layer precipitates. It seems more likely that the long-term effect of the pipe material on biofilm formation is negligible.

8 MICROBIAL PROCESSES

The plug-flow nature of the flow in drinking water pipes subdues suspended bacteria to a rapid washout. The typical retention time in the distribution network do not allow for many cell divisions of suspended bacteria. There are only two ways a bacterial strain can maintain itself within the network, namely by: 1) attachment to the surfaces or 2) being constantly feed to the system. The attached bacteria may subsequently re-enter the suspended phase by detachment (Figure 11).

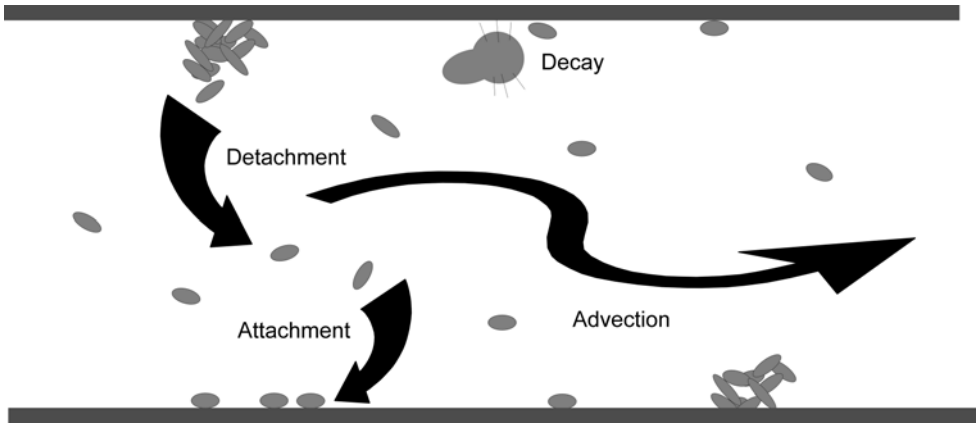

Figure 11. Dynamics of biofilm formation.

It is important to realise that no single factor (see previous chapter) is responsible for the water deterioration. The dynamics of the drinking water networks is extremely complex. The aftergrowth may shift between being a) kinetically controlled, when retention time is low, and b) capacity controlled, when retention time is high. This means that an increased substrate concentration does not necessarily lead to increase in the water phase bacterial numbers observed, while the combination of an increased substrate concentration and a high retention time most likely will.

Ideally a model of drinking water quality changes in distribution systems caused by microbial activity should include all the factors listed in the previous chapter and a detailed knowledge of the dynamics of processes they influence. The processes to be considered are:

- Bacterial growth
- Attachment/detachment
- Decay (grazing, lysis etc.)
- Hydrolysis

8.1 Bacterial growth

The microbial growth is mainly controlled by a) the amount and availability of the substrate, b) the temperature, and c) the presence of inhibitory compounds. The Monod expression (Monod, 1949) is widely used to describe the bacterial growth (Equation 1), since it generally gives at good description of the observed relation between the observed growth and substrate concentration.

$$\frac{dX}{dt} = \mu_{max} \frac{S}{S + K_s} X \qquad (1)$$

X: biomass; t: time; μ_{obs}: observed growth rate; μ_{max}: maximum growth rate; S: substrate concentration; K_s: half saturation constant.

However, the determination of parameter values based on experiments is difficult since the maximum growth rate (μ_{max}) and half saturation (K_s) constants are confounded (Sommer, 1997). Parameter values obtained from the literature are often very specific to the experimental conditions under which they were obtained, and such values should therefore always be cautiously used.

For the temperatures relevant to drinking water the bacterial growth is expected to increase at higher temperatures according to the Arrhenius equation.

8.2 Attachment/Detachment

The transfer of cells from water phase to surface occurs as a combination of passive (diffusive) transport and active cell motion (Mueller, 1996). At the surface the cells may irreversibly attach themselves facilitated by excretion of EPS. Early colonisers may precondition the surface, which may subsequently raise the attachment rate. For the passive adsorption, equilibrium between the amount of bacteria in the water phase and on the surfaces can be reached within a few hours, while the active microbial attachment is slower. The main factors influencing the rate of attachment are the hydraulics, the nature of the substratum and the amount and properties of the microorganisms in the system.

The detachment designates the transfer process of microorganisms from surface to water phase. The process is a combined biological and physical process where all the mechanisms involved have yet to be discovered. An incomplete list of the biological factors influencing detachment includes cell motility within the biofilm, synthesis and release of EPS weakening/degrading enzymes (Boyd and Chackrabarty, 1994), cell growth rate (Sawyer and Hermanowicz, 1998), grazing activity and cell death/lysis. The detachment rate from the biofilm is affected by the turbulence of the system (i.e. the shear stress). However, the biofilm seem to adapt to the hydraulic conditions by

changing its structure (Van Loosdrecht et al., 1995). Peyton and Characklis (1993) showed that biofilm formed under different but constant levels of shear stress had a similar detachment rate. In drinking water distribution networks the situation is dissimilar due to the ever-changing flow rates of the pipes. Few studies have considered the effect and dynamics on the detachment of biomass when exposed to flow variations.

In drinking water systems, the attachment process is mainly important when considering the effects of a pathogen intrusion. The detachment is very important in controlling the amount of biofilm present, but the kinetics is very complex since it is a combination of an odd number of processes. Nonetheless, several different empirically based mathematical expressions have been proposed for practical purposes (as cited in Peyton and Characklis, 1993).

8.3 Decay

The death of a bacterium can be inflicted by environmental stress, such as starvation, presence of biocides, temperature changes etc. Other factors of importance may be grazing by protozoa and viral infections. Cell lysis where the cell components are released to the surroundings will occur at some point after the cell death. The lysed cell components may later be hydrolysed and used for growth by other bacteria.

In different aquatic systems, grazing activity has been shown to influence the morphological structure, the taxonomic composition and the physiological state of the bacterial communities (Hahn and Höfle, 2001). Several studies have reported protozoan grazing activity to affect the dynamics of microbial growth in drinking water (Pedersen, 1990; Sibille et al., 1997). In a study by Kalmbach et al. (1997a) grazing horizons were observed, but the importance of this activity in drinking water has yet to be determined. Quantification of the grazing activity poses a serious challenge to researchers, since a) the protozoa are sparsely distributed in the biofilm making quantification difficult, and b) the methods currently available for determination of specific rate of grazing are extremely tedious.

In marine oligotrophic systems, virus infections have been shown to have a great influence on the mortality of suspended bacteria (Guixa-Bixereu et al., 1999). No studies has to the author's knowledge documented the presence of an indigenous viral-loop in non-contaminated drinking water, nevertheless viruses is probably present, though their role and importance remains unknown.

8.4 Hydrolysis

The natural organic matter (NOM) in drinking water originating from groundwater consists mainly of high molecular weight compounds like humic and fulvic substances (Grøn et al., 1996), which are not readily available to the bacteria. A study in drinking water by Hem and Efraimsen (2001) has shown that AOC was mainly related to the fraction of NOM with a low molar weight. Thus the conversion of organic matter by hydrolysis may be an important process, since it reduces the size of the molecules making them more available to the bacteria. The hydrolysis may occur throughout the distribution network, but little is known about the dynamics and the importance of these processes. The process is potentially very important in non-chlorinated systems, since controlling the amount of substrate may be a difficult task if these are generated rapidly within the distribution network.

9 DETERMINATION OF BIOFILM GROWTH

Since our knowledge of the dynamics of the individual processes in drinking water generally is very limited, we need to rely on simplified expressions, which may be used for practical purposes.

Determination of biofilm growth is generally difficult. On a micro-scale the biofilm is in a constant state of change, thus the concept of microbial growth has to relate to the biofilm on a larger scale. Four different approaches can be applied in order to quantify the bacterial growth of drinking water biofilms.

- Direct measurement
- Biofilm formation rate
- Mass-balances
- Detachment rate

The different approaches are described in the following chapter.

9.1 Direct measurement

Several methods have been proposed as a direct measurement of microbial growth rate. For drinking water systems frequency of dividing cells (FDC) technique and the leucineincorporation seem to have the greatest potential.

The bacterial growth rate of a natural bacterial community can theoretically be determined directly from a microscopic slide by observing the fraction of cells in the later stage of cell division, this can be directly related to the growth rate of the community (Hagström et al., 1979). However, FDC is difficult to apply in slow growing bacterial communities like drinking water, since the number of dividing cells is very low compared to the total number of bacteria.

As previously described the leucine incorporation technique can give a rough estimate of the bacterial growth (Boe-Hansen, 2002c). The rate of rate of leucine incorporation varies greatly between different microbial communities. This means that the calculating an absolute value for the growth rate requires a good determination of the conversion factor between cell production and leucine incorporation, which may be very difficult to achieve. The potential of the method lies in detecting changes in a specific community rather than determination of an absolute value for the growth rate.

9.2 Biofilm formation rate

Perhaps the most commonly used method for determining biofilm growth is measurement of the biofilm formation rate on virgin surfaces. Practically, the measurement is performed by successive harvesting test surfaces after different periods of drinking water exposure (Figure 12).

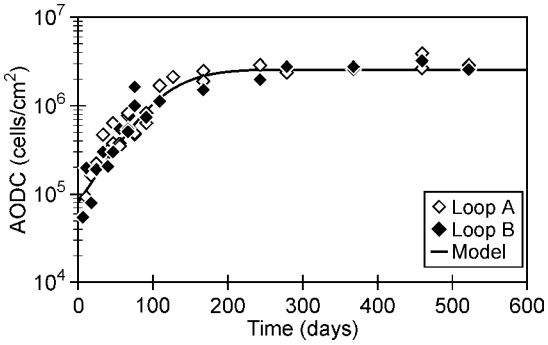

Figure 12. Biofilm formation in a model distribution system (AODC) (Boe-Hansen, et al. 2002b).

The approach does not allow for segregation of the attachment, growth and decay processes, which are pooled into a net growth expression. Boe-Hansen et al., (2002b) achieved a good estimation using a simple expression for the bacterial growth and detachment (Equation 2 and Figure 12).

$$\frac{dX}{dt} = \mu_{obs} X - k X^2 \qquad (2)$$

X: Attached total bacteria (AODC). t: Time. μ_{obs}: Observed growth rate. k: Detachment rate constant.

In studies by Pedersen (1990) and Van der Kooij et al. (1995) the biofilm formation rate in drinking water has been determined in a similar way.

9.3 Mass-balances

At stationary phase the growth of bacteria are balanced by biofilm detachment, grazing and cell decay. The true stationary phase is only possible if all extrinsic factors remain constant, which is practically impossible in biological systems. A constant selection occurs within the biofilm, which will favour new organisms whenever the environmental conditions change (Gilbert, 2000). The stationary biofilm is in a state of dynamic equilibrium sometimes referred to as a quasi- or pseudo stationary phase.

At quasi-stationary phase the bacterial net production will be continuously transferred into the bulk water phase. By measuring the increase in suspended bacteria after a period of time, the net production can be estimated. The net growth rate can be calculated from the bacterial production if the total amount of bacteria is known. It is important to notice that the method does not allow for distinguishing between attached and suspended growth. Equation 3 can be applied in calculating the net growth rate in a closed batch system as used by Boe-Hansen et al. (2002b).

$$\mu_{obs} = \frac{1}{t_{batch}} \log\left(\frac{M_{total,1}}{M_{total,0}}\right) \qquad (3)$$

μ_{obs}: Observed growth rate, $M_{total,0}$, $M_{total,t}$: Total biomass at the start (0) and after (t) the batch experiment, t_{batch}: Time of batch experiment.

Mass-balances have been used to estimate growth rates in drinking water systems by Van der Wende et al. (1989) and Block et al. (1993).

9.4 Detachment rate

A possible way to measure the bacteria growth rate in drinking water is by measuring the release rate of bacteria from the biofilm. The biofilm can be radioactively labelled by amending the drinking water with an easily degradable carbon source like benzoic acid. The radioactivity of the surfaces will steadily decline due to detachment of microorganisms and respiration (endogenous or grazing) (Equation 4).

$$\text{Loss rate} = \text{detachment} + \text{respiration} \qquad (4)$$

Although the detachment rate at stationary phase is constant, the specific radioactivity of the cells being released decreases. The decrease is mainly caused by growth of the microorganisms, where every cell cycle halves the specific radioactivity (if endogenous respiration is neglected). Therefore the radioactivity being released from the stationary biofilm will decrease at a rate equivalent to the net growth rate of the active bacteria in the biofilm. The method has been applied by Boe-Hansen et al. (2002c).

9.5 Growth determinations

The drinking water bacterial communities are generally slow growing, due to the low temperature and the limited nutrient supply. Table 7 shows different growth rates observed in drinking water by various methods.

Table 7. Observed bacterial growth rates in drinking water systems.

Substrate	Method	Parameter	Growth (d^{-1})	Chlorine	Reference
Surface water	Mass balance	HPC	0.060*	No	Van der Wende et al. (1989)
Surface water	Formation rate	AODC	0.063	Yes	Pedersen (1990)
Surface water	Mass balance	AODC	0.041	Yes	Block et al. (1993)
Groundwater	Formation rate	ATP	0.013*	No	Van der Kooij et al. (1995)
Surface water	Formation rate	ATP	0.013*	Yes	Van der Kooij et al. (1995)
Groundwater	Formation rate	AODC	0.030	No	Boe-Hansen et al. (2002b)
Groundwater	Mass balance	AODC	0.049	No	Boe-Hansen et al. (2002c)
Groundwater	Detachment	^{14}C	0.013	No	Boe-Hansen et al. (2002c)
Groundwater	Direct measurement	Leu.incorp AODC	0.008	No	Boe-Hansen et al. (2002c)

*Approximation (my calculation)

Apart from the detachment rate method, the other methods provided an average net growth rate, which means we are unable to distinguish between a small but fast growing community and a large but slow growing community.

The bacterial growth rate appears to be higher during the initial biofilm growth phase compared to stationary phase. A study of a pure culture biofilm has shown that the growth rate was decreasing as the microcolonies grew older, which was most likely a response to depletion of nutrient in the vicinity of the individual microcolony (Sternberg et al., 1999). In the oligotrophic environment such as drinking water, it has been proposed that the bacterial interaction with surfaces allows the bacteria to utilise nutrients absorbed and concentrated at the surfaces thus enables the biofilm bacteria to grow faster during the early growth phase. The continued growth may deplete the nutrient pool at the surface, which will eventually reduce the growth rate (Gilbert, 2000). A reduced growth rate may lead to a sudden decline in biomass, which has been observed in activated carbon filters by Servais et al. (1994). In distribution networks, a decline in bacterial growth rates during the biofilm maturation has been observed by Boe-Hansen et al. (2002c).

10 PROJECT CONCLUSIONS

In the following chapter the major project conclusions of the thesis is outlined. The conclusions are mainly based on the results from the experimental work described in the papers enclosed as appendices.

Model distribution systems are generally suitable for studies of the interaction between biofilm and water phase. The model system, which was designed, constructed and tested in this project, allowed for detailed studies of biofilm and water phase interaction at conditions realistic to drinking water.

The microbial growth is to a great extent dependent on the level of microbial available organic substrate. Different concentrations have been proposed as a limit for biological stability. However, the present study showed that significant growth occurred even at very low concentrations (AOC > 5 µg-ac/L). The attached bacterial growth rate was determined to 0.030 d^{-1} during biofilm formation. At least 200 days was needed in order to establish quasi-stationary conditions at 2.6×10^6 cells/cm^2.

A good correspondence between bacterial growth and AOC removal was observed.

The AOC consumption was rapid, since AOC was generally depleted to below the detection limit (<0.1 µg ac-C/L) within 24 hours of retention time. The yield of the bacteria growing on AOC was determined to 1.0×10^7 cells/µg, which was similar to literature values observed for pure culture bacteria isolated from drinking water.

The microbial net growth of distribution system is normally affiliated with the activity of the biofilm bacteria. However, the present studies at low nutrient conditions showed the suspended bacteria to account for a significant fraction of the total bacterial growth. The activity of the suspended bacteria was generally higher in terms of growth rate, culturability, ATP content, and leucineincorporation than the biofilm bacteria.

Cultivation methods have been widely applied by researchers and water supplies as a measure of microbial biomass in drinking water. In the present studies the HPC method was unfit for quantification of the indigenous microbial activity in low nutrient drinking water systems, this was primarily due to low culturability of the bacterial community and a observed high variability between replicates.

The biofilm changed its properties in terms of culturability and ATP content during the biofilm maturation. In the fully matured system, the overall bacterial growth rate (attached and suspended) of the model distribution system was 0.049 d^{-1}. The growth

of the biofilm appeared to be lower in the mature biofilm, compared to the growth rate during biofilm formation.

Few studies have considered the degradation of specific carbon compounds at concentrations environmentally realistic. This study showed benzoic acid to be degraded at a rate close to the AOC removal. This suggests that the AOC consisted mainly of low molecular weight organic compounds. Amendment with benzoic acid was a useful tool for studies of the microbial substrate turnover in drinking water systems. The yield of the biofilm degradation of benzoic acid was low ranging from 0.024 to 0.034 g C/g C and the yield of the suspended bacteria was significantly higher.

The presence of an indigenous mature biofilm in the drinking water distribution network may be beneficial to the hygienic quality of the drinking water, since a) the survival capability of pathogenic bacteria is reduced, b) the capacity for bacterial production is reduced, because biofilm bacteria (having a small yield) compete for substrate with suspended bacteria (having a high yield).

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APPENDICES

Appendix A Boe-Hansen, R., Albrechtsen,H. -J., Arvin, E. and Jørgensen, C. Development of a model distribution system for studies of microbial activity and changes in drinking water quality. Manuscript in preparation. 2002a.

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